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Lactobacillus plantarum IS-10506 activates intestinal stem cells in a rodent model

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RESEARCH ARTICLE

Abstract

This study investigated the probiotic effect of *Lactobacillus plantarum* IS-10506 in activating and regenerating leucine-rich repeat-containing G-protein-coupled receptor (Lgr)5- and B lymphoma Moloney murine leukaemia virus insertion region (Bmi)1-expressing intestinal stem cells in rodents following *Escherichia coli* serotype 055:B5 lipopolysaccharide (LPS) exposure. Male Sprague-Dawley rats (n=64) were randomised into control (KN), LPS (KL), probiotic + LPS (KL-Pr), and sequential probiotic + LPS + probiotic (KPR-7L) groups. Microencapsulated *L. plantarum* IS-10506 (2.86×10^{10} cfu/day) was administered via a gastric tube once daily for up to 7 days, and LPS (250 µg/kg body weight) was administered via a gastric tube on the first day of the experiment to all but the KN group. On day 3, 4, 6, and 7, four rats per group were sacrificed, and Lgr5, Bmi1, extracellular signal-regulated kinase (ERK), and β-catenin expression in the ileum was assessed by immunohistochemistry. LPS treatment reduced Lgr5 ($P\leq0.05$) and Bmi1 (P=0.000) levels in intestinal epithelial cells, whereas probiotic treatment increased levels of Lgr5 (KPR-7L, P=0.008) and Bmi1 (KL-Pr, P=0.008; and KPR-7L, P=0.000). Lgr5 expression was upregulated in the KL-Pr group on day 3, 4, 6, and 7 (P=0.056). Additionally, ERK levels were elevated in Bmi1- and Lgr5-expressing cells in rats treated with probiotics (KL-Pr and KPR-7L), whereas β-catenin levels were increased in Lgr5-expressing cells from KPR-7L rats and in Bmi1-expressing cells from KL-Pr and KPR-7L rats on day 3 and 4. These results demonstrated that the probiotic *L. plantarum* IS-10506 activated intestinal stem cells to counter inflammation and might be useful for maintaining intestinal health, especially when used as a prophylactic agent.

Keywords: probiotic, intestinal stem cell, mucosal damage, regeneration, lipopolysaccharide

1. Introduction

Probiotics are live microorganisms that confer health benefits when consumed in adequate amounts (FAO/WHO, 2002), with one of its function is reducing duration of infectious diarrhoea in children (Allen *et al.*, 2010). Recovery from diarrhoea is related to the repair of intestinal epithelium damage, and probiotic administration was found to mitigate ileal mucosal damage and inflammation, as well as to alter cytokine profiles in *Salmonella typhimurium*-infected mice (Castillo *et al.*, 2013). *Lactobacillus plantarum* IS-10506 is a probiotic isolated from dadih, a fermented buffalo milk from Sumatra Island (Akuzawa and Surono, 2002). Additionally, *L. plantarum* IS-10506 and IS-20506 increase the expression of intestinal brush border structural proteins, such as galectin-4, myosin-1a, occludin, and zona occludens-1 (Ranuh, 2008).

The surface of intestinal epithelial cells consists of villi and crypts, where a high proliferation rate is balanced with apoptosis (Yen and Wright, 2006). Stem cells proliferate into multipotent progenitor cells that differentiate into absorptive enterocytes, mucin-producing goblet cells, hormone-producing enteroendocrine cells, and Paneth cells (Clevers, 2013; Scoville *et al.*, 2008; Yeung *et al.*, 2011). The intestinal stem cell population consists of columnar base label-retaining cells (LRCs) expressing leucine-rich repeat-containing G-protein-coupled receptor (Lgr)5 and B lymphoma Moloney murine leukaemia virus insertion region (Bmi)1 (Barker *et al.*, 2007; Yan *et al.*, 2011). Lgr5-

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positive stem cells continuously divide to regenerate intestinal epithelial tissue. Signalling pathways involved in stem cell activation and proliferation include Wnt, which is marked by high β-catenin activity (Crosnier *et al.*, 2006), and epidermal growth-factor receptor (EGFR), which induces stem cell proliferation via mitogen-activated protein kinase (MAPK)/ extracellular signal-regulated kinase (ERK) signalling (Karim and Rubin, 1998). Proliferation is followed by differentiation and maturation during the repair of damaged mucosa (Umar, 2010).

In this study, we investigated the probiotic effect of *L. plantarum* IS-10506 on protein expression in stem cells during intestinal inflammation induced by *Escherichia coli* O55:B5 lipopolysaccharide (LPS) in a rodent model.

2. Materials and methods

Animals

Ethical approval was obtained from the Ethics Committee (Animal Care and Use Committee) of Veterinary Medicine School, Airlangga University (Surabaya, Indonesia). Male Sprague-Dawley rats (12-weeks old, 100-120 g; n=64) were randomised into control (KN), LPS (KL), probiotic + LPS (KL-Pr), and sequential probiotic + LPS + probiotic (KPR-7L) groups, each of which was subdivided into four subgroups (n=4 rats each) that were sacrificed on day 3, 4, 6, and 7. Rats were allowed to adapt for 1 week prior to the start of experiments. Sterile water was administered via a gastric tube for 14 days in control (KN) rats. The KL group was treated with *E. coli* O55:B5 LPS on day 1 and sterile water on the remaining 13 days. The KL-Pr group received LPS on day 1 and *L. plantarum* IS-10506 on day 2 until the time of sacrifice. The KPR-7L group was given *L. plantarum* IS-10506 for 6 days before LPS administration, followed by the probiotic until the time of sacrifice. At the end of the experiment, the ileum was dissected for analysis. Rats were examined daily for morbidity and other symptoms of ill health, such as reduced activity level, abnormal evacuation, and decreased body weight.

Probiotic

Microencapsulated *L. plantarum* IS-10506 (GenBank accession no. DQ860148) was used as a probiotic. The probiotic was packed in an aluminium foil sachet at the Pharmacy Installation of Dr. Soetomo Hospital (Surabaya, Indonesia) and administered via a gastric tube once daily for 6 days after LPS administration in the KL-Pr group, or for 6 days before and after LPS administration in the KPR-7L group. Probiotic viability was assessed 1 week prior to the intervention. The probiotic powder was administrated by dissolving in 1.5 ml sterile water at a dose of 2.9×10^{10} cfu/day.

Lipopolysaccharides

E. coli O55:B5 LPS (L2880; Sigma-Aldrich, St. Louis, MO, USA) was used at a dose of 250 µg/kg body weight (diluted with 0.9% NaCl in a 10:1 ratio). LPS was orally administered via a gastric tube on day 1 of the study in all but the KN group.

Immunohistochemistry

The ileum was cleaned and fixed in 10% formalin buffer solution, followed by dehydration, clearing, and embedding. Tissue sections were probed with antibodies against Lgr5 (sc-135238; Santa Cruz Biotechnology, Dallas, TX, USA) and Bmi1 (sc-10745; Santa Cruz Biotechnology) to evaluate protein expression in intestinal stem cells, and against β-catenin (sc-1496; Santa Cruz Biotechnology) and ERK (sc-94; Santa Cruz Biotechnology) to detect activation of Wnt and MAPK signalling pathways. Samples were observed with a light microscope (CX21; Olympus, Tokyo, Japan) and photographed with an ILCE6000 camera (Sony, Tokyo, Japan). The number of immunopositive cells was determined by counting the mean number of cells in 20 random fields at 1000× magnification.

Statistical analysis

Differences between groups were evaluated by one-way analysis of variance for data with a normal distribution and with the Mann-Whitney and Kruskal-Wallis tests for non-normally distributed data (two and more than two groups, respectively). Significance was set at $P<0.05$.

3. Results

Probiotic administration promotes the regeneration of intestinal tissue damaged by lipoplysaccharides

Rats treated with LPS showed fewer Lgr5-expressing cells on day 3 (KL, $P=0.02$; KL-Pr, $P=0.02$; and Kpr-7L, $P=0.02$) as compared to the KN group, indicating that the intestinal stem cell population was reduced by LPS-induced inflammation. However, *L. plantarum* IS-10506 administration increased the number of Lgr5-positive cells in the KPr-7L group ($P=0.008$) (Figure 1A), but the increase required pre-treatment with the probiotic prior to LPS administration, as the KL-Pr group showed no difference in the number of Lgr5-positive cells when compared to the KL group. Lgr5 expression decreased in the KL group ($P=0.037$) from day 4 to 6, but increased in the KL-Pr group ($P=0.046$) from day 4 to 6, and in the KPr-7L group, an increase was observed starting at day 4 ($P=0.019$) and persisted until the end of the experiment. The increase in the number of Lgr5-expressing cells on day 7 in rats pre-treated with *L. plantarum* IS-10506 indicated that probiotic promoted the recovery of intestinal mucosa.

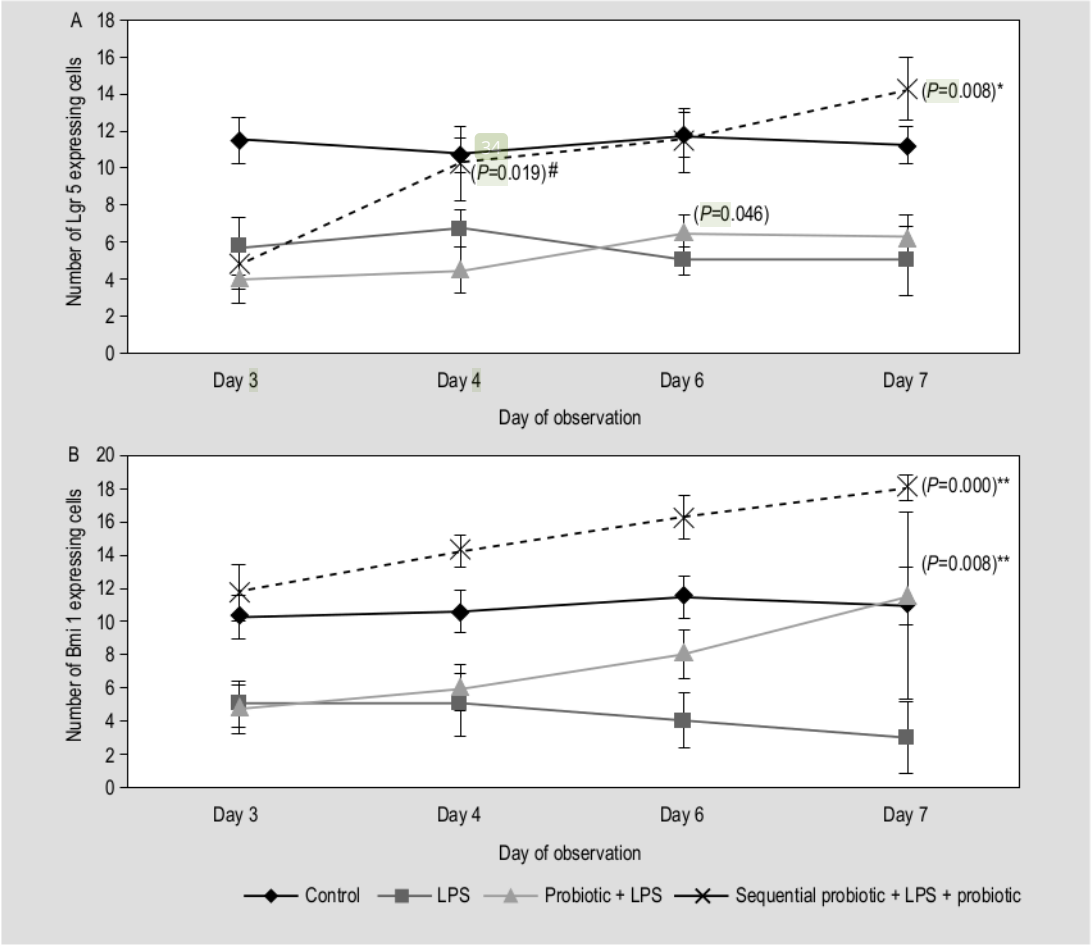

Figure 1. Changes in Lgr5 (A) and Bmi1 (B) expression over time. + Significant change in expression from day 3 to 4; # Significant change in expression from day 4 to 6; * Significant change in expression from day 6 to 7; ** Significant change in expression from day 3, 4, 6, and 7.

On day 3, the number of Bmi1-expressing cells was lower in the KL (P=0.019) and KL-Pr (P=0.019) groups when compared to the KN group; moreover, there was no significant difference in Bmi1-positive cell number between the KPr-7L and KN groups (P=0.180), suggesting that probiotic administration prior to LPS treatment had a protective effect. The increase in the number of Bmi1-positive cells was observed starting on day 7 in the KL-Pr group (P=0.008), and on day 3 in the KPr-7L group (P=0.000), which persisted until day 7 (Figure 1B).

Probiotic administration activates intestinal stem cells

We observed fewer ERK/Lgr5 double-positive cells in the KL group as compared with the KN group on day 3 (P=0.018). However, the KPr-7L group showed a higher number of ERK/Lgr5-expressing cells than that observed in the KN group (P=0.020). Additionally, ERK expression increased in the KL-Pr (P=0.006) and KPr-7L (P=0.000) groups over time (Figure 2A), with upregulation detected on day 4 (P=0.028) and 6 (P=0.028) in the KL-Pr group, and on day 4 (P=0.019) in the KPr-7L group.

The number of ERK/Bmi1 double-positive cells was comparable in the KL and KN groups on day 3 (P=0.647); however, the number increased in rats receiving probiotic treatment (KL-Pr, P=0.019; and KPr-7L, P=0.019). An overall increase in ERK activation was observed in the KL-Pr (P=0.003) and KPr-7L (P=0.034) groups; however, ERK activation decreased between day 3 and 4 in both groups (KL-Pr, P=0.017; and KPr-7L, P=0.013), and between day 6 and 7 (P=0.046) in the KL-Pr group, but increased between day 4 and 6 (P=0.047) in the KPr-7L group (Figure 2B).

We observed fewer β-catenin/Lgr5 double-positive cells in the KL (P=0.017) and KL-Pr (P=0.019) groups as compared

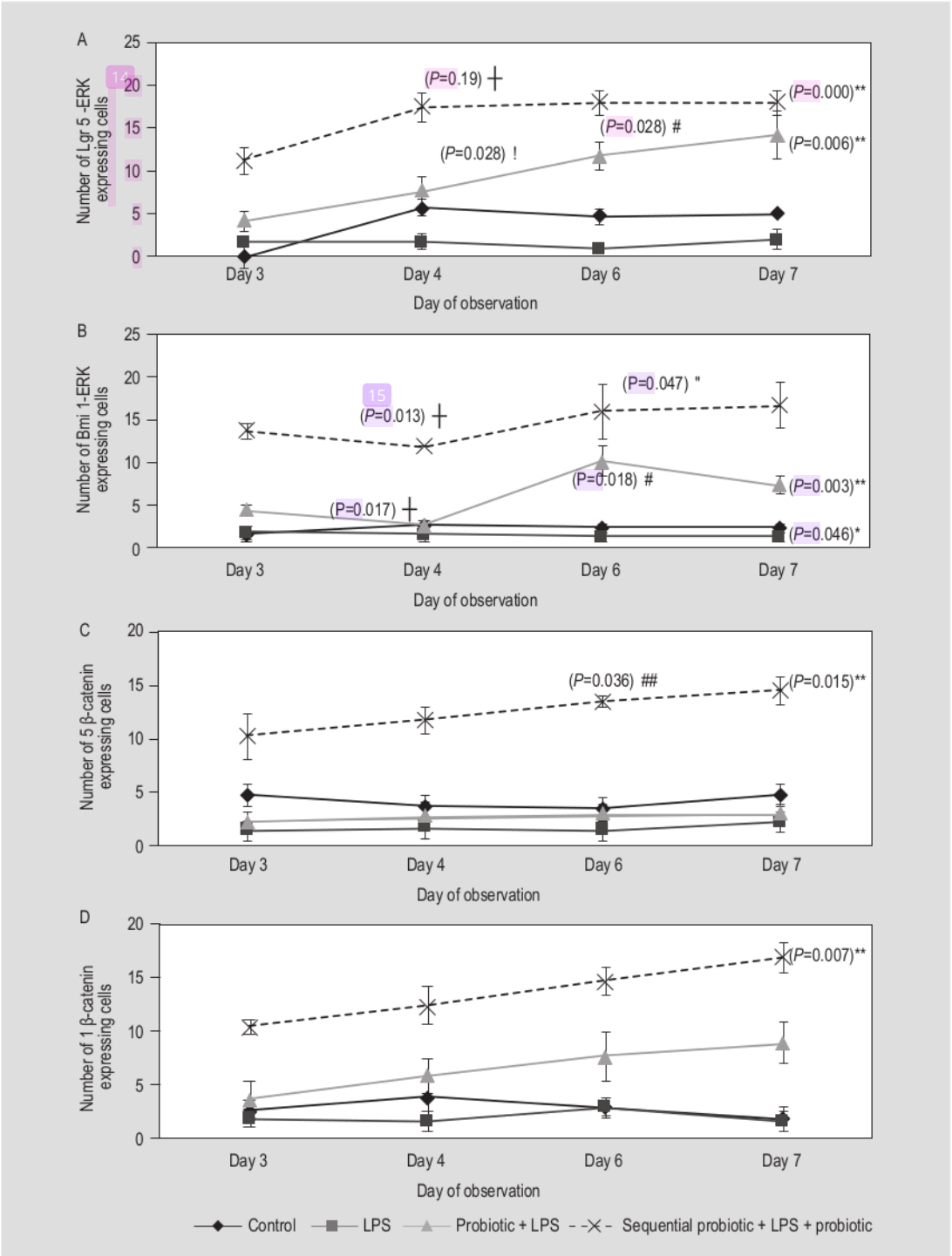

Figure 2. (A, B) Extracellular signal-regulated kinase (ERK) and (C, D) β-catenin expression in cells expressing Lgr5 (A, C) and Bmi1 (B, D) as a function of time. + Significant change in expression from day 3 to 4; # Significant change in expression from day 4 to 6; * Significant change in expression from day 6 to 7; ** Significant change in expression from day 3, 4, 6, and 7; ## Significant change in expression from day 3, 4, and 6.

with the KN group on day 3. Probiotic administration increased the number of cells expressing β-catenin/Lgr5 only in the KPr-7L group (P=0.036; Figure 2C) relative to those in the KN group, and the immunoreactivity increased over time in the KPr-7L group (P=0.015; Figure 2C).

At day 3, the number of β-catenin/Bmi1 double-positive cells did not differ between the KL (P=0.278) and KL-Pr (P=0.369) groups as compared with that observed in the KN group; however, the number of cells expressing both β-catenin and Bmi1 was higher in the KPr-7L group relative to the KN group (P=0.019). Additionally, β-catenin activation increased over time in the KPr-7L group (P=0.007; Figure 2D).

4. Discussion

Epithelial cells of the small intestine exhibit self-renewal capacity and cells are replaced every 3 to 5 days in mice. Consistent with a previous study (Ranuh, 2008), we found that LPS administration caused damage to the ileal mucosa, as shown by a decrease in the number of Lgr5- and Bmi1-expressing cells. However, intestinal stem cell regeneration occurred in rats that were not treated with *L. plantarum* IS-10506, based on the presence of Lgr5-expressing cells in the KL group. This agreed with a previous finding that a subset of crypt cells expressing high levels of sex-determining region Y-box 9 (i.e. LRCs) co-express activated intestinal stem cell markers, such as Lgr5 (Roche *et al.*, 2015). Probiotics activate cells by inducing the Toll-like receptor (TLR)-2 receptor in Lgr5-expressing cells (Scheeren *et al.*, 2014), leading to an increase in enterocyte proliferation (Hörmann *et al.*, 2014). *Lactobacillus* spp. (*L. rhamnosus* GG, *L. acidophilus* and *L. casei*) administration also showed cytoprotective effects against radiation-induced intestinal injury, which was dependent upon TLR-2 signalling (Ciorba *et al.*, 2012). In the present study, we found that probiotic pre-treatment abrogated LPS-induced decreases in Bmi1-positive intestinal stem cell number relative to control animals after 3 days. This represents the first evidence that probiotic administration protects Bmi1-positive cells under conditions of inflammation.

Under normal conditions, homeostasis of the intestinal mucosa is maintained by Lgr5-expressing cells, whereas Bmi1-positive cells can compensate for the loss of Lgr5-positive cell function (Barker *et al.*, 2007; Carlone and Breault, 2012; Montgomery *et al.*, 2011; Shivdasani, 2014; Tian *et al.*, 2011). Additionally, cells expressing Bmi1 are more resistant to radiation-induced injury than those expressing Lgr5 and contribute to the clonal expansion of epithelial cells during tissue regeneration (Yan *et al.*, 2011). Therefore, higher numbers of Bmi1-positive cells are expected to increase the regenerative potential of intestinal mucosa.

The recovery of intestinal mucosa following injury or infection depends not only upon stem cell activation but also on coordination between stem cell proliferation and differentiation. Various signalling pathways contribute to this process, including signalling associated with Janus kinase/signal transducer and activator of transcription, EGFR, bone-morphogenetic protein, Wnt, and Notch (Buchon *et al.*, 2010). In the present study, we found that β-catenin was downregulated and upregulated in the KL and KL-Pr groups, respectively, as compared with the KN group. Moreover, we also observed increases in the number of β-catenin/Lgr5 double-positive cells. A previous study reported that β-catenin phosphorylation increased in mouse colon epithelial cells following administration of *Salmonella*, leading to a decrease in β-catenin activity and target gene expression (Duan *et al.*, 2007). Importantly, in animals treated with probiotics, we observed more β-catenin/Bmi1 double-positive cells relative to the KN group; with the highest number observed in the KPr-7L group. This suggested that the probiotic had a greater effect on quiescent stem cells than activated stem cells.

We observed that ERK expression was higher and time-dependent in the KPr-7L group as compared with the KN group. In contrast, ERK was down regulated in the KL and KL-Pr groups relative to the KN group. These results were consistent with those reported in a study demonstrating that increased TLR-2 signalling activates ERK1/2 and AKT pathways, which play important roles in epithelial cell proliferation (Hörmann *et al.*, 2014). We also found that the number of ERK/Bmi1 double-positive cells was higher in rats treated with probiotic as compared with the KN group, and the number was highest in the KPr-7L group, indicating that *L. plantarum* IS-10506 had a preventative effect against inflammation-induced epithelial tissue injury.

Furthermore, *L. plantarum* IS-10506 had a greater effect on EGFR signalling than on Wnt signalling. We speculated that prophylactic probiotic administration increased ERK expression, activated EGFR signalling, and thereby enhanced intestinal epithelial cell proliferation.

5. Conclusions

Our results suggested that *L. plantarum* IS-10506 increased the size of the Lgr5- and Bmi1-expressing intestinal stem cell pool and induced the activation of these cells based on elevated ERK and β-catenin expression to mitigate intestinal mucosal injury caused by inflammation. These findings demonstrated that *L. plantarum* IS-10506 is a potentially effective therapy, especially when used prophylactically, for the maintenance of gastrointestinal health.

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PAGE 1

PAGE 2

PAGE 3

PAGE 4

PAGE 5

PAGE 6